RAPID COMMUNICATION

Monoclonal Antibodies Specific for Melanocytic Tumors Distinguish Subpopulations of Melanocytes

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The authors have generated monoclonal antibodies to an extract of melanoma. When tested on a variety of fixed, embedded sections of malignant tumors, one antibody (HMB-45) reacted with 60 of 62 melanomas and none of 168 nonmelanomas (carcinomas, lymphomas, and sarcomas). The antibody reacts with junctional nevus cells but not intradermal nevi, and recognizes fetal and neonatal melanocytes but not normal adult melanocytes. This antibody thus demonstrates absolute specificity for melanocytic tumors and thus has great utility for the surgical pathologist in distinguishing among poorly differentiated tumors of uncertain origin. It also identifies differences among populations of melanocytes which may be useful in understanding the biology of and interrelationships between these cells. (Am J Pathol 1986, 123:195–203)

MALIGNANT MELANOMA constitutes approximately 1% of all cancers, and its incidence is increasing at a rate exceeded only by that of lung carcinomas. Melanomas can be diagnostic problems for surgical pathologists because they may present as a metastatic tumor, and they can be difficult to distinguish from other neoplasms such as carcinomas, lymphomas, and sarcomas. Traditional methods of distinguishing melanomas from other neoplasms include argyrophil stains or the demonstration of DOPA oxidase by histochemical techniques. However, the utility of these techniques is limited because the DOPA method is preferably used on postfixed cryostat sections, and reducing methods such as Masson–Fontana silver stains also react with substances other than melanin, such as lipofuscin and argentaffin cell granules.[2] More recently, an immunocytochemical approach using antibodies to S-100 protein has been suggested as a more definitive test for melanoma. Although quite sensitive, antibodies to S-100 are not melanoma-specific; they react with a diverse set of tumors, including Schwann cell tumors,[3] chondroblastomas, chondrosarcomas, osteosarcomas,[4] malignant gliomas,[5] Langerhans cells, and other histiocytic tumors,[6] as well as a subset of carcinomas.[7]

Monoclonal antibody technology has allowed the development of antibodies with predetermined tissue specificities, including those that can be used as diagnostic reagents by virtue of their reactivity in fixed, embedded tissues. We have described here a monoclonal antibody, HMB-45, that is absolutely specific, and highly sensitive for melanoma and junctional nevi in fixed, embedded tissue.

Materials and Methods

Generation of Antibody

A portion of an axillary lymph node containing pigmented melanoma metastases was used as an immunogen and a screening tissue. A 0.3-cu cm portion of tissue was minced finely in phosphate-buffered saline (PBS). A BALB/c mouse received intraperitoneal injections weekly for 3 weeks, with a booster injection at Week 5. Three days after the final injection, the spleen

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was removed and a fusion procedure was performed with NS-1 cells as previously described.[8] Hybridomas were screened by immunocytochemistry on Carnoy's-fixed, paraffin-embedded sections of the same melanoma that was used as an immunogen. Sections were placed on Teflon-coated, multiwelled glass slides (Meloy Laboratories, Burlingame, Calif), deparaffinized through graded alcohol baths, and overlayed with supernatant fluid (50–100 μl) from each of the original 288 wells. A standard avidin–biotin immunoperoxidase technique was used to identify positive wells (Vector Laboratories, Burlingame, Calif). Hybridomas producing antibodies that recognized melanoma but not endothelial cells, lymphocytes, or stromal cells in the lymph node were cloned by methods previously described.[8] Three monoclonal antibodies designated HMB-18, HMB-45, and HMB-50 were obtained by this procedure. All three antibodies displayed similar reactivity on the tumor, although HMB-45 displayed the strongest and most consistent immunostaining and was used for further studies.

Cell Culture

The Mel-1 cell line was established from a portion of the lymph node used to generate HMB-45. Tissue was minced, rinsed in PBS, and subjected to brief collagenase treatment. The cells were initially grown on gelatin-coated dishes but are now routinely passaged on uncoated dishes in Dulbecco's modified Eagle's medium with 10% fetal calf serum. The cells are heavily pigmented and have been maintained in continuous culture for 18 months.

Selection of Tumors

Tissue from 18 cases of primary cutaneous melanoma and 44 cases of metastatic or recurrent melanoma (subcutis, retina, lymph node) were examined on a prospective basis from case material at the University Hospital and Swedish Hospital, Seattle, Washington. The diagnosis of melanoma was established in each case by 1) histologic review of all primary melanomas by three pathologists not involved in this study; 2) reactivity with a panel of tissue-specific monoclonal antibodies in which all melanomas displayed a common vimentin-postive, cytokeratin-negative, T-200-negative phenotype[9]; 3) reactivity with polyclonal antibodies to S-100 protein[10]; 4) ultrastructural analysis, in a few cases with unusual histology, to confirm the presence of premelanosomes. Only those 13 primary melanomas fixed in formalin were vimentin-negative; vimentin is not reliably identified in formalin-fixed tissue, and thus histologic study and S-100 immunocytochemistry alone were used to verify these 13 diagnoses. Additionally, 2 cases of atypical melanocytic hyperplasia, 5 dysplastic nevi, 9 nevi, 4 blue nevi, 6 Spitz tumors, 1 case of lentigo maligna, and normal skin were tested.

Also tested were 168 cases of nonmelanoma malignant tumors (40 sarcomas, 31 lymphomas, 91 carcinomas, 6 gliomas). All lymphomas were positive with monoclonal antibodies to T-200 antigen (Dako Laboratories, Santa Barbara, Calif), and all carcinomas tested were positive with at least one of two anti-cytokeratin monoclonal antibodies.[9] All sarcomas were vimentin-positive. In cases where the subcategorization of a sarcoma was in doubt, ultrastructural analysis revealed no evidence of premelanosome formation or other features to suggest a diagnosis of melanoma.

Immunocytochemistry (Tissue Sections)

Most tissues were fixed in methacarn fixative (methanol-Carnoy's)[11]; some were fixed in 4% formaldehyde in phosphate buffer (see Table 1). After overnight fixation and paraffin embedding, sections were cut and deparaffinized in sequential alcohol solutions and incubated with the monoclonal antibody ascites fluid at a dilution of 1:1000. Tissue fixed in formaldehyde was subjected to brief incubation with Pronase (Calbiochem-Behring, San Diego, Calif) (0.01% in PBS) at room temperature for 30 minutes prior to antibody incubation. All tissues were also incubated with rabbit antibodies to S-100 protein (Dako, 1:1000 dilution), and all methacarn-fixed tissue was also incubated with monoclonal antibodies to vimentin (43βE8)[12] and cytokeratins (35βH11 and 34βE12).[12] Antibody localization was via the avidin–biotin immunoperoxidase technique as described previously[9] with nickel chloride modification of the diaminobenzidine (DAB) reaction.[13]

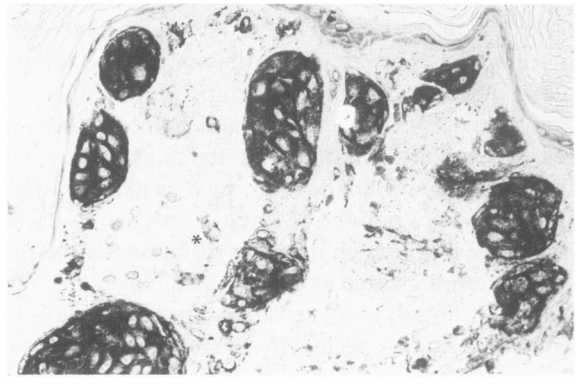

Figure 1—Avidin–biotin immunoperoxidase preparation of superficial spreading melanoma with antibody HMB-45. Antibody delineates the extent of tumor cells. Melanophages (*) appear granular in this black and white reproduction, but in the original slide, with nickel intensification of DAB (see text), their brown melanin is easily distinguishable from the black reaction product in the tumor cell population. (× 100)

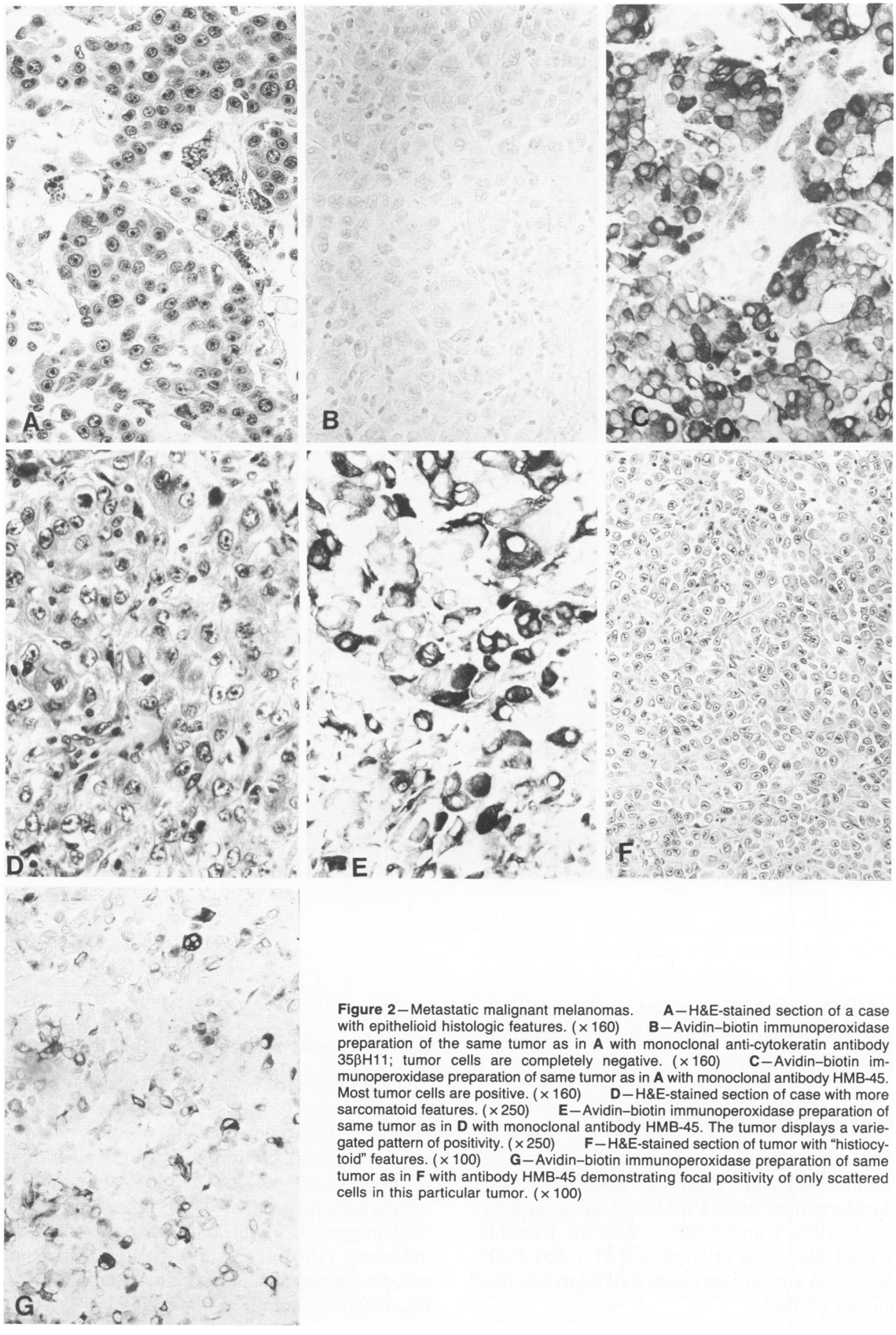

Figure 2—Metastatic malignant melanomas. **A**—H&E-stained section of a case with epithelioid histologic features. (×160) **B**—Avidin–biotin immunoperoxidase preparation of the same tumor as in **A** with monoclonal anti-cytokeratin antibody 35βH11; tumor cells are completely negative. (×160) **C**—Avidin–biotin immunoperoxidase preparation of same tumor as in **A** with monoclonal antibody HMB-45. Most tumor cells are positive. (×160) **D**—H&E-stained section of case with more sarcomatoid features. (×250) **E**—Avidin–biotin immunoperoxidase preparation of same tumor as in **D** with monoclonal antibody HMB-45. The tumor displays a variegated pattern of positivity. (×250) **F**—H&E-stained section of tumor with "histiocytoid" features. (×100) **G**—Avidin–biotin immunoperoxidase preparation of same tumor as in **F** with antibody HMB-45 demonstrating focal positivity of only scattered cells in this particular tumor. (×100)

Table 1—Malignant Tumors

Tumor	HMB-45		S-100		Anti-vimentin 43βE8		Anti-cytokeratin (34βE12 or 35βH11)	
	+	−	+	−	+	−	+	−
Melanomas								
Primary*	18	0	18	0	5	13‡	0	18
Metastatic,† recurrent	42	2	44	0	44	0	0	44
Carcinomas†								
Breast	0	14	1	13	0	14	14	0
Colon	0	10	0	10	0	10	10	0
Pancreas	0	2	0	2	0	2	2	0
Thyroid	0	2	0	2	1	1	2	0
Endometrium	0	5	0	5	5	0	5	0
Ovarian Cystadenocarcinoma	0	12	8	4	6	6	12	0
Salivary gland, all types	0	6	4	2	6	0	6	0
Squamous cell, all sites	0	10	0	10	0	10	10	0
Mesothelioma	0	7	0	7	6	1	7	0
Renal cell	0	5	1	4	4	1	5	0
Lung, all types	0	8	2	6	2	6	8	0
Basal cell, skin	0	3	0	3	0	3	3	0
Cloacogenic	0	1	0	1	0	1	1	0
Islet cell	0	3	0	3	0	3	3	0
Embryonal and seminoma	0	3	0	3	0	3	1	2
Lymphomas†								
All types	0	31	1	30	0	31	0	31
Sarcomas†								
Angiosarcoma	0	4	1	3	4	0	0	4
Epithelioid	0	1	0	1	1	0	1	0
Ewing's	0	1	0	1	0	1	0	1
Kaposi's	0	2	0	2	2	0	0	2
Leiomyosarcoma	0	3	0	3	3	0	0	3
Liposarcoma	0	1	1	0	1	0	0	1
Malignant fibrous histiocytoma	0	5	0	5	5	0	0	5
Neurogenic sarcoma	0	6	6	0	6	0	0	6
Chondrosarcoma	0	2	2	0	2	0	0	2
Rhabdomyosarcoma	0	1	0	1	1	0	0	1
Synovial sarcoma	0	2	0	2	2	0	2	0
Retinoblastoma	0	1	0	1	0	1	0	1
Sarcoma, NOS	0	11	3	8	7	4	0	11
Glioma, NOS	0	6	6	0	6	0	0	6

* Thirteen were fixed in formalin; 5 were fixed in Carnoy's or methanol–Carnoy's fixative.
† All specimens were fixed in Carnoy's or methanol-Carnoy's fixative.
‡ All 13 vimentin-negative tumors were those fixed in formalin (see text).

Nickel chloride makes the reaction product black instead of brown, which allows it to be more easily distinguished from melanin.

Immunofluorescence (*In Vitro* Cells)

Indirect immunofluorescence was performed on cells grown on multiwell slides (Meloy). The cells were fixed in 2% paraformaldehyde in PBS for 5 minutes and permeabilized in 1% Triton X-100 in PBS for 1 minute. Ascites fluid was used at dilutions of 1:200–1:400. Fluoresceinated anti-mouse antibody (Tago) was used at a dilution of 1:40.

Results

Immunocytochemistry on Malignant Tumors

HMB-45 reacted with 97% of the melanomas tested (18/18 primary tumors and 42/44 metastatic lesions) (Table 1). Both pigmented and nonpigmented tumors were recognized. Quantitation of the reaction ranged from focal reactivity (less than 10% of tumor cells positive) to uniform positivity (>80% of all tumor cells positive). In general, "epithelioid" melanoma cells were more uniformly HMB-45-positive than more sarcomatous, spindle-shaped melanoma cells. Examples of the immunostaining are given in Figures 1 and 2.

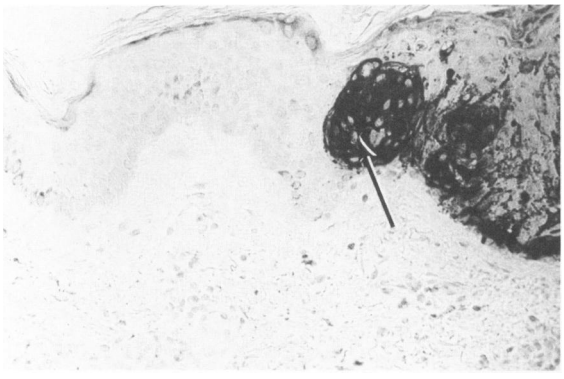

Figure 3—Avidin–biotin immunoperoxidase preparation of edge of primary site of superficial spreading melanoma with antibody HMB-45. Antibody, while positive on the intraepithelial nests of melanoma cells (*arrow*), is nonreactive with the adjacent normal melanocyte population (*left*). (×100)

The melanomas were cytokeratin-negative and vimentin-positive (Table 1). Additionally, anti-S-100 was positive on all melanomas, including the 2 tumors that did not react with HMB-45.

Malignant tumors of epithelial, lymphoid, glial, and mesenchymal origin were all negative, including 91 carcinomas, 31 lymphomas, and 46 sarcomas (Table 1). In every case, all tumor cells were HMB-45-negative. However, in 3 basal cell carcinomas and one basaloid cloacogenic carcinoma there were rare HMB-45-positive cells with dendritic morphologic features scattered throughout the tumor. The origin of these HMB-45-positive cells is unclear, but they may represent a "reactive" melanocytic population within the tumor.

Immunocytochemistry on Normal Skin

When assayed on normal human skin, or on normal skin adjacent to melanocytic lesions, HMB-45 failed to react with melanocytes (Figure 3). The degree of pigmentation did not appear to affect antibody reactivity, because skin from black individuals was equally negative (data not shown). Reactivity with normal melanocytes could not be induced by increasing 10-fold the antibody concentration or by pretreatment of the tissue with proteolytic enzymes (trypsin, Pronase). Occasionally, however, the pigment cells of the hair follicle were positive with HMB-45. Additionally, melanocytes in fetal skin were variably positive (data not shown).

Immunocytochemistry on Benign Melanocytic Lesions

Benign nevi manifested two patterns of reactivity, depending on their location within the skin. Junctional nevi and the junctional components of compound nevi were positive, whereas intradermal nevi and the dermal components of compound nevi were consistently negative (Figures 4 and 5). All cases of Spitz tumors and atypical melanocytic hyperplasia were also positive (Table 2).

Immunofluorescence Localization of HMB-45 Antigen

The immunocytochemistry results suggest that HMB-45 recognizes a cytoplasmic antigen(s). To pursue this matter further, we performed immunofluorescence staining on cells derived from the original tumor. Mel-

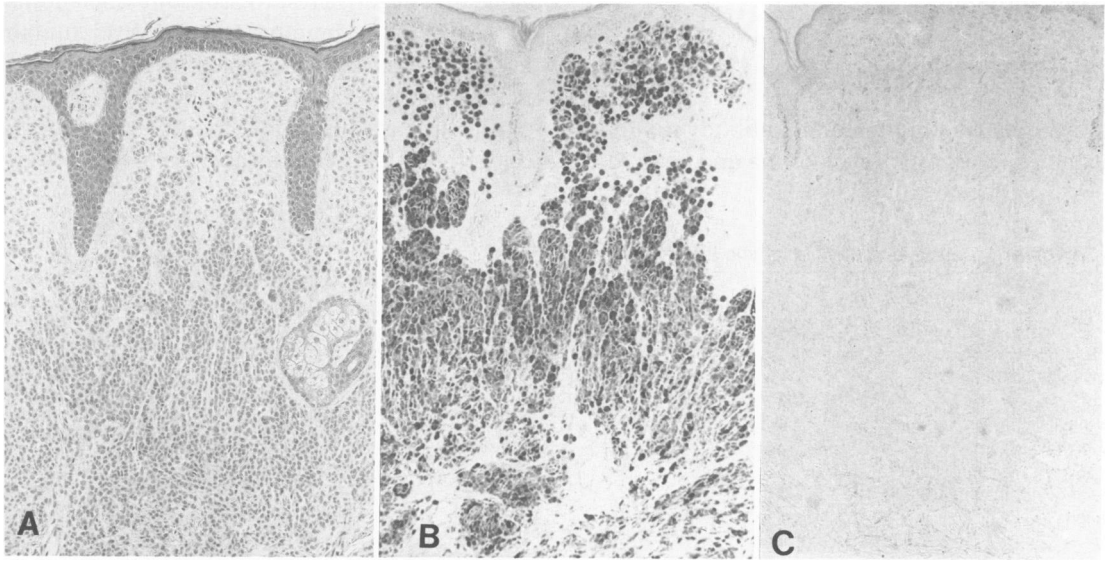

Figure 4—Intradermal nevus. **A**—H&E-stained section. **B**—Avidin–biotin immunoperoxidase preparation with polyclonal antibodies to S-100 protein. **C**—Avidin–biotin immunoperoxidase preparation with monoclonal antibody HMB-45. S-100 antibodies react with entire population of dermal nervus cells; antibody HMB-45 is nonreactive with all cells. (×100)

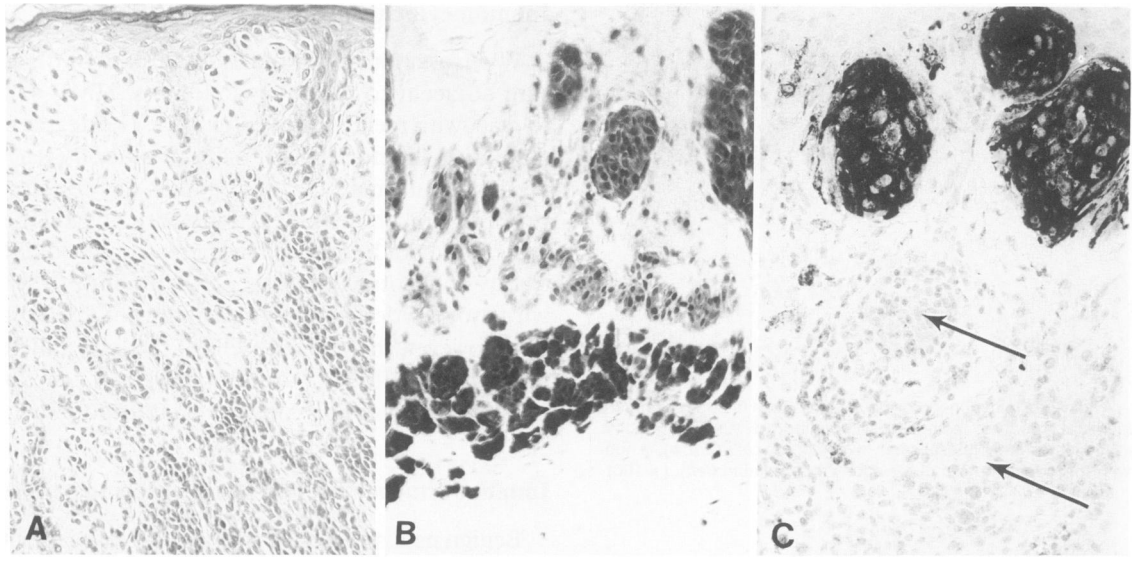

Figure 5—Compound nevus. **A**—H&E-stained section. **B**—Avidin–biotin immunoperoxidase preparation with polyclonal antibodies to S-100 protein. **C**—Avidin–biotin immunoperoxidase preparation with monoclonal antibody HMB-45. S-100 antibodies react with the entire population of intradermal and junctional nevus cells; antibody HMB-45 reacts with the junctional component but is nonreactive with the intradermal nevus cells (*arrows*). (×160)

1 cells exhibited an intracytoplasmic granular fluorescence pattern (Figure 6). Nonmelanoma cells, including human fibroblasts, Hep3B (hepatocellular carcinoma), and A431 (an epidermoid carcinoma line), did not react with the antibody; increasing the antibody concentration 10-fold did not result in reactivity with nonmelanoma cells (data not shown). The immunofluorescence results are thus consistent with assays on fixed embeded tissue in that only melanoma cells are recognized by the antibody.

Discussion

We have generated a monoclonal antibody that displays absolute specificity for melanoma and nevocytic lesions and is therefore an excellent reagent for use in surgical pathology. When assayed on fixed, paraffin-embedded tissue, HMB-45 recognized 97% of the melanomas tested and none of the other 168 malignant tumors. Based on this data, the predictive value of a positive test (ie, the proportion of positive tests that actually identify the disease) on a given malignant tumor would be 100% (PV = [number true positive]/[number true positive + number false positive]). The predictive value of a negative test to "rule out" melanoma would be 98.4% (PV + [number true negative]/[number false negative + number true negative]). The antibody also reacts with Spitz tumors, atypical melanocytic hyperplasias, and the junctional components of benign nevi, but fails to react with intradermal nevus cells. Normal

Table 2—Benign and Premalignant Melanocytic Lesions

		HMB-45		S-100		
	N*	+	−	+	−	Fixation
Normal skin melanocytes	8	0	8	8	0	5M,† 3F‡
Blue nevi	4	1	3	3	1	2M, 2F
Intradermal nevi	6	0§	6	6	0	4M, 2F
Compound nevi	3	3‖	3	3	0	2M, 1F
Dysplastic nevi	5	5	0	5	0	5F
Atypical melanocytic hyperplasia	2	2	0	2	0	2F
Lentigo maligna	1	1	0	1	0	1M
Spitz tumor	6	6	0	6	0	5M, 1F

* N, number of cases.
† M, methacarn.
‡ F, formaldehyde.
§ One case showed slight positivity of most superficial dermal nevus cells.
‖ Junctional component only positive.

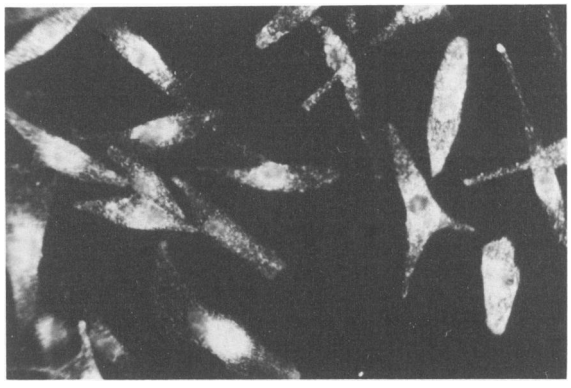

Figure 6—Indirect immunofluorescence demonstrating reactivity of monoclonal antibody HMB-45 on melanoma cell line Mel-1 (see text). The antibody decorates the cells with a granular, cytoplasmic pattern. (×250)

Table 3—Summary of Reactivity of Tumors with Monoclonal Antibodies

	Anti-cytokeratin*	Anti-vimentin†	Anti-T200‡	HMB45
Carcinoma	+	–§	–	–
Lymphoma	–	–	+	–
Melanoma	–	+	–	+
Sarcoma (nonmelanoma)	–	+	–	–

* Monoclonal antibodies 35βH11 or 34βE12.[9]
† Monoclonal antibody 43βE8.[9]
‡ Monoclonal antibody (Dako).
§ Carcinomas of selected sites may co-express vimentin.[12]

tissues are negative, including adult melanocytes. We do not know why 2 melanomas failed to react with HMB-45. A number of melanomas exhibited striking regional or segmental positivity, and it is possible that we did not sample enough of the tumor to detect the positive regions.

Clinical Uses of HMB-45

The most obvious use for the antibody is in cases of undifferentiated malignant tumors where it is difficult to distinguish among melanoma, sarcoma, carcinoma, and lymphoma. The absolute specificity of the antibody for melanocytic lesions and the absence of false-positive reactions with other malignant tumors allow one to identify undifferentiated neoplasms that react with HMB-45 as melanomas with certainty. For example, of the 44 metastatic melanomas described in this study (Table 1), 3 represent cases in which melanoma was totally unsuspected. To classify undifferentiated neoplasms, we routinely employ a panel of tissue-specific monoclonal antibodies; the panel includes anti-cytokeratin, anti-vimentin, anti-T-200 antibodies, and antibody HMB-45. Each neoplasm in Table 3 manifests a unique pattern of reactivity with this panel. Carcinomas are cytokeratin-positive, lymphomas are T-200-positive, and melanomas are HMB-45-positive.

HMB-45 is also useful in lymph node dissections in identifying microscopic clusters of metastatic melanoma cells (Figure 7). The total absence of background staining has allowed the identification of small foci of tumor cells within nodes that were thought to be negative. A retrospective study is now being done to determine how often small tumor foci are missed on routine examination of sections stained with hematoxylin and eosin (H&E). Formalin-fixed tissue can be used in this study, provided that Pronase treatment is carried out prior to incubation with antibody. Reactivity on formalin-fixed tissue is more variable than that observed on methacarn-fixed material, but some formalin-fixed sections react as intensely as methacarn-fixed sections.

Unfortunately, HMB-45 is not useful in distinguishing benign and malignant melanocytic proliferations because it recognizes junctional nevi, Spitz tumors, and atypical melanocytic hyperplasias.

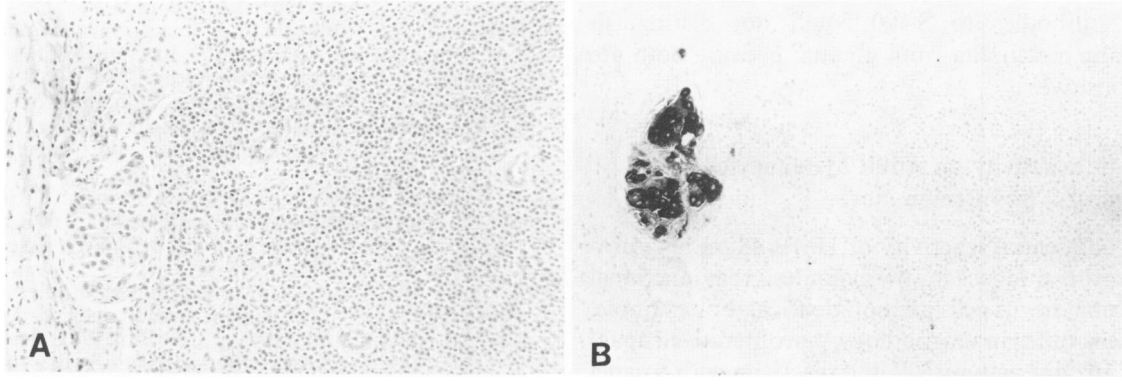

Figure 7—Axillary lymph node containing a single focus of metastatic melanoma in the subcapsular sinus. **A**—H&E-stained section. **B**—Serial section demonstrating a nest of tumor cells positive with monoclonal antibody HMB-45, as demonstrated with the avidin–biotin immunoperoxidase method. (×100)

Comparison With Other Anti-Melanoma Monoclonal Antibodies

A number of laboratories have isolated monoclonal antibodies to melanoma-associated antigens, but their utility as melanoma-specific diagnostic reagents is uncertain; many also react with nonmelanocytic tumors, and fixation and paraffin embedding destroys antigenicity in others.[14-21] The latter is the case with the majority of anti-melanoma antibodies which have been generated to cell surface antigens. Recently, another group has characterized monoclonal antibodies that discriminate between melanomas and nevocellular nevi.[22] HMB-45, however, appears unique in combining the properties of 1) absolute specificity for melanocytic cells and 2) applicability to fixed, embedded surgical pathology material. Other markers such as neuron-specific enolase and histochemical markers such as the silver stain for argyrophil granules ("melanin stain") have markedly less specificity and sensitivity. The gamma isozyme of enolase ("neuron-specific" when expressed as a homodimer) is by no means specific for melanoma, benign melanocytic lesions, or even for neurons, as the name suggests.[23] Empirical "melanin stains" are not helpful when the tumor is amelanotic, and they are positive on many other neoplasms, such as other APUD tumors.[2]

Comparison of HMB-45 With Anti-S-100

Antibodies to S-100 protein have been found by many to be a sensitive marker for melanoma[10]; and in our hands, anti-S-100 stained every melanoma tested. However, it is clear that S-100 is not melanoma-specific, because many nonmelanocytic tumors express this antigen, including schwannomas, cartilagenous tumors, salivary gland tumors, gliomas, and even a subset of breast carcinomas.[7] Thus, undifferentiated tumors arising from these tissues could be mistaken for melanoma on the basis of reactivity with anti-S-100 alone. In the brain, antibodies to S-100 could not distinguish metastatic melanoma from glioma, because both are S-100-positive.

HMB-45 Reactivity on Adult Melanocytes, Fetal Melanocytes, and Benign Nevi

The differential reactivity of HMB-45 on nevi provides further evidence for the hypothesis that junctional and dermal nevus cells are not identical. For example, these cells differ in morphology,[24] proliferative capacity,[25,26] enzyme activity,[27,28] and reactivity with a panel of monoclonal antibodies to melanoma-associated antigens.[29] The differential reactivity of fetal and adult melanocytes also implies differences between these cells and is consistent with the observation that these cells express different antigens.[30] Thus, it will be important to identify the antigen(s) recognized by this antibody to better understand the differences among these cells.

We do not know the reasons for this differential reactivity. It is probably not due to the presence or absence of melanosomes because these organelles are present in adult melanocytes and dermal nevi.[31,32] One possible explanation is that the antibody recognizes epitopes expressed only in proliferating melanocytes, both benign and malignant. This idea is consistent with the hypothesis that nevus cells proliferate at the dermal epidermal junction and then "drop off" and become quiescent within the dermis.[25,33,34] It also would explain why fetal melanocytes, which are proliferative cells, react while quiescent adult melanocytes are unreactive. If this is true, then HMB-45 may be very useful in *in vitro* studies of proliferating melanocytes.

We are currently trying to identify the cellular structure(s) and antigen(s) recognized by the antibody. The immunofluorescence data suggest that the antigen(s) is cytoplasmic, and we are trying to confirm this by immunoelectron microscopy. Biochemical analysis has proven difficult because the antibody does not work on "Western blots." We are now attempting immunoprecipitation experiments using ^{35}S-methionine-labeled melanoma cells in culture.

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